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TRANSLOCATION OF PROTONS AND POTASSIUM IONS ACROSS THE MITOCHONDRIAL MEMBRANE OF RESPIRING AND RESPIRATION-DEFICIENT YEASTS

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SUMMARY

1. Mitochondria from aerobically grown wild-type yeast *Saccharomyces cerevisiae*, incubated anaerobically in a K^+-containing medium are impermeable to protons. Permeability to protons was induced by uncoupler in the presence of valinomycin. Swelling in potassium acetate and energy-dependent K^+ transport induced by valinomycin were similar to those of mammalian mitochondria except that the extent of the volume changes or of K^+ uptake was considerably smaller with yeast mitochondria.

2. Mitochondria isolated from a cytoplasmic respiration-deficient "petite" mutant were also quite impermeable to H^+. As in wild-type yeast mitochondria, valinomycin enhanced the passive permeability for K^+ in the mutant mitochondria and, together with an uncoupler, induced proton permeability. However, no ATP-dependent K^+ uptake could be demonstrated.

3. In contrast to wild-type yeast mitochondria, mitochondria from the respiration-deficient mutant exhibited neither uncoupler-sensitive $^{32}P_i$–ATP exchange nor a decrease in 8-anilino-1-naphthalene sulphonic acid, sodium salt (ANS) fluorescence on addition of ATP. It is concluded that although the permeability characteristics of the mutant mitochondria are similar to those of normal yeast mitochondria, the terminal segment of the energy-transfer system is rendered non-functional by the cytoplasmic mutation.

INTRODUCTION

The resolution of the components of the mitochondrial membrane and the reconstitution of a functional membrane from the purified components[1] have provided considerable insight into the mechanism of energy coupling in mitochondria. As an alternate approach, various deficiencies can be introduced into the mitochondrial membrane *in vivo* by cytoplasmic or nuclear mutations[2]. Multiple mutational lesions can be superimposed in a cell to further increase the extent of deficiencies in the mito-

Abbreviations: ANS, 8-anilino-1-naphthalene sulphonic acid, sodium salt; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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chondria[3]. Ultimately, such efforts may help in the reconstitution of the system of oxidative phosphorylation by *in vivo* and *in vitro* recombinations or by addition of isolated components to the mutant mitochondria.

This genetic approach to mitochondrial energy coupling is limited to a few biological species and, thus far, yeast *Saccharomyces cerevisiae* has been used almost exclusively. Although the properties of wild-type yeast mitochondria have been extensively studied in recent years, little is known about their osmotic and permeability properties and about the translocation of ions and substrates across their membranes. Translocation of some ions, in particular of hydrogen[4], potassium[5] and calcium[6] seems to be intimately linked to the energy-coupling mechanism of the mitochondrial membrane.

In this paper, H^+ and K^+ permeabilities of mitochondria isolated from wild-type yeast and a respiration-deficient mutant are described. Evidence is presented that the mutation to respiratory deficiency ("petite" mutation) affects the energy-transfer system of yeast mitochondria although impermeability to protons and permeability to K^+ in the presence of valinomycin were still observed in the mutant cells.

MATERIALS AND METHODS

Chemicals

The source of chemicals used is indicated in parentheses. 8-Anilino-1-naphthalene sulphonic acid, sodium salt (ANS)[1] (K & K), was recrystallized from water three times; bovine serum albumin (Pentex); carbonylcyanide *m*-chlorophenylhydrazone (CCCP), valinomycin and EDTA (Calbiochem); oligomycin, antimycin A, 2-mercaptoethanol, D-mannitol, D-sorbitol, sodium ATP and sodium ADP (Sigma). Atractyloside was a gift from Dr. A. Bruni. Sorbitol solutions were purified by stirring with Dowex 50 (H^+-form) for 2 h and filtered.

Yeast culture and isolation of mitochondria

The diploid strain *Saccharomyces cerevisiae* DT XII and the corresponding cytoplasmic respiration-deficient strain DT XIIa were used. The cells were grown aerobically under conditions used in previous studies with 0.5 % glucose as carbon source and harvested late in the logarithmic growth phase. Protoplasts and mitochondria were prepared by a modification of a previously published procedure[7]. The washed cells (1 g dry weight) were suspended in 20 ml of 0.5 M 2-mercaptoethanol buffered with 0.1 M Tris–HCl (pH 9.3). After incubation for 5 min at 30°, the suspension was centrifuged, the cells washed with 40 ml of 1.5 M sorbitol (pH 7.0) and finally suspended in a final volume of 10 ml in a solution containing 1.35 M sorbitol, 1 mM EDTA and 10 mM citrate phosphate buffer (pH 5.8). About 200 mg of Helicase (Industrie Biologique Française, Gennevilliers, France) dissolved in a minimal volume of 1.5 M sorbitol, or 1.4 ml of Glusulase (Endo Laboratories, Garden City, N. Y.) were added and the suspension was incubated at 30° with occasional stirring. After about 90 % of the cells had been converted into osmotically fragile forms as monitored by observation in a phase-contrast microscope, the suspension was centrifuged at room temperature and the protoplasts washed two times at pH 7.0 with 40 ml of 1.5 M sorbitol and once with 40 ml of 0.75 M sorbitol–0.4 M mannitol–0.1 % bovine serum albumin. For homogenization, the protoplasts were suspended in 30 ml of a solution containing 0.6 M mannitol, 2 mM EDTA and 0.2 % serum albumin (pH 7.0), homogenized in an

overhead blendor (Lourdes Instruments) for 10 sec at top speed, and then centrifuged at 1500 × *g* for 10 min. The pellet was resuspended in 5 ml of the same solution with a hand-operated Potter–Elvehjem homogenizer and the suspension was centrifuged at 1500 × *g* for 10 min. The supernatants from the two centrifugations were pooled and centrifuged at 8000 × *g* for 10 min. The sedimented mitochondria were resuspended with a hand-operated glass homogenizer in 5 ml of the homogenization medium, from which serum albumin had been omitted. The suspension was centrifuged at 1200 × *g* for 5 min, the pellet discarded, and the mitochondria sedimented from the supernatant at 18000 × *g* for 10 min. The surface of the sediment was gently washed with a small amount of 0.6 M mannitol and the mitochondria suspended in 0.6 M mannitol and stored up to 5 h at 0° at about 50 mg protein per ml. The yield is approximately 15 to 20 mg mitochondrial protein per g of dried yeast cells. This procedure has been successfully used for the preparation of promitochondria[11], and mitochondria from other strains. Differences in the susceptibilty to the snail gut enzyme have been encountered with different strains and with different times of harvesting. Accordingly, appropriate adjustments in the time of incubation with the snail enzyme should be made.

Analytical methods

Protein was determined by the biuret method[8]. Fluorescence enhancement of ANS in mitochondria[9,10] was measured in an Eppendorf fluorimeter with a 313 + 366-nm filter for excitation and a 400–3000-nm filter for emission lights. Respiration was measured with a Clark oxygen electrode at 25° in a 1.5-ml cell of the Gilson oxygraph. Respiratory control was calculated as ratio of the respiration rate in the presence of ADP to the rate established after exhaustion of ADP.

Swelling of mitochondria was followed by determining absorbance at 520 nm. $^{32}P_i$–ATP exchange[11], ATPase[7] glyceraldehydephosphate dehydrogenase[12] and proton translocation[13,14] were measured as described. Buffering capacities of the medium and mitochondria, half-times of pH equilibration across the mitochondrial membrane, proton conductance coefficient and H^+/O ratios, were determined according to published procedures[13,14]. Changes in K^+ concentrations in the mitochondrial suspension were measured with a Beckman cation-sensitive electrode 39047. The reference electrode of the Thomas combination electrode served as the reference electrode.

RESULTS AND DISCUSSION

Factors affecting the quality of isolated yeast mitochondria

In the past, studies of yeast mitochondria were considerably hampered by the lack of adequate isolation procedures. Although mitochondria have been prepared from yeast protoplasts[7,15,16] or from cells with partially digested cell walls[17], these preparations have shown relatively low respiratory control and high Mg^{2+}-dependent ATPase activity which was insensitive to atractyloside[18].

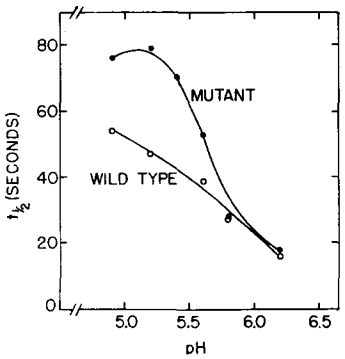
In the present study, we have observed that the properties of the starting protoplasts is important for the quality and quantity of the resulting mitochondria. The use of purified sorbitol and careful control of pH yielded mitochondria with respiratory control values of 5 with α-ketoglutarate and 4 with pyruvate *plus* malate. In the absence of Mg^{2+}, the ATPase activity was very low and virtually insensitive to un-

couplers and K^+ *plus* valinomycin. In the presence of Mg^{2+}, the ATPase activity was still high, oligomycin-sensitive and only slightly inhibited by atractyloside. These properties made the study of ATP-driven proton translocation[19] virtually impossible with yeast mitochondria.

Proton permeability of the yeast mitochondrial membrane

At neutral pH, the pH equilibration across the mitochondrial membrane was too rapid to allow recording. The half-time ($t_{1/2}$) of pH equilibration across the membrane of wild-type yeast mitochondria increased with decreasing pH down to pH 4.9. A similar pH dependence of pH equilibration was observed with mitochondria isolated from the respiration-deficient mutant (Fig. 1).

The outer and inner buffering powers of mitochondria from both wild-type yeast and the respiration-deficient mutant exhibited a pH dependence (Fig. 2) similar to that observed with rat-liver mitochondria[13] suggesting the presence of similar types of dissociable groups. With decreasing pH (between pH 6.2 and 4.9) both the inner and outer buffering powers increased in parallel with $t_{1/2}$ the proton conductance coeffi-



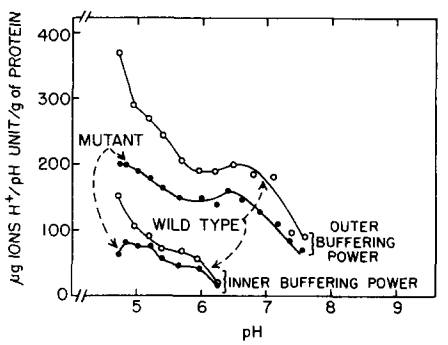


Fig. 1. Half-time of the pH equilibration across the membrane of yeast mitochondria. Yeast mitochondria (3.5 mg/ml) were suspended in 0.4 M mannitol, 0.15 M KCl and 3.3 mM glycylglycine. The suspension was flushed with N_2 for 10 min. After a pulse of anaerobic HCl, the half-time of the pH equilibration was calculated from secondary pH rise according to MITCHELL AND MOYLE[13,14].

Fig. 2. pH dependence of the outer and inner buffering power of yeast mitochondria. The incubation conditions were the same as described in Fig. 1. Outer and inner buffering powers were determined as described by MITCHELL AND MOYLE[13,14].

TABLE I

PROTON CONDUCTANCE COEFFICIENTS (C_M) OF YEAST MITOCHONDRIA AT DIFFERENT pH VALUES

pH	C_M (*μg ions H^+ per sec per pH unit per g protein*)	
	Wild-type yeast mitochondria	*Respiration-deficient mitochondria*
4.9	1.07	0.51
5.2	0.99	0.48
5.4	—	0.43
5.6	0.90	0.48
5.8	1.16	0.85
6.2	1.15	0.67

cient (μg H^+ ions per sec per pH unit per g mitochondrial protein (see ref. 13)) was thus virtually independent of pH (Table I). When the mitochondrial suspension was adjusted to pH 7.5 or higher and titrated down to 4.9, the pH equilibration was much faster even at the lower pH's indicating damage to the mitochondria at alkaline pH.

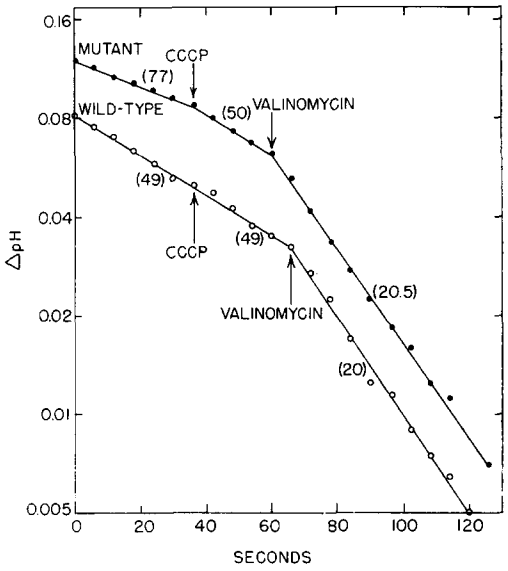


Fig. 3. Effect of CCCP and valinomycin on the pH equilibration across the yeast mitochondrial membrane. Yeast mitochondria (5.0 mg protein/ml for the wild-type; 2.7 mg protein/ml for the mutant) were incubated as described in Fig. 1 at pH 6.4. The pH of the suspension was lowered approximately 1.2 units by a pulse of anaerobic HCl. The kinetics of the subsequent slow pH rise were followed. CCCP (6 nmoles/ml) or valinomycin (0.8 μg/ml) were added at the arrows. The figures at the lines indicate half-times of the pH equilibration in sec.

TABLE II

H^+/O RATIOS DURING SUBSTRATE OXIDATION IN YEAST MITOCHONDRIA

An oxygen pulse was given to an anaerobic suspension containing 0.4 M mannitol, 0.15 M KCl, 3.3 mM glycylglycine, wild-type yeast mitochondria (3.0 mg protein/ml in Expt. I and 2.6 mg protein/ml in Expt. II), and additions as indicated below. H^+/O ratios were calculated according to MITCHELL AND MOYLE[13]. The starting pH in all experiments was 6.5.

Expt. No.	*Substrate*	*Additions*	*H^+/O ratio*
I	2 mM sodium succinate	—	3.2
		0.2 μg/ml valinomycin	4.0
	4 mM sodium citrate	—	3.8
		0.2 μg/ml valinomycin	3.9
II	1.25 mM sodium pyruvate + 1.25 mM sodium malate	—	3.3
		0.2 μg/ml valinomycin	4.0
		4 mM $CaCl_2$	3.1
		4 mM $CaCl_2$ + 0.2 μg/ml valinomycin	4.1
		0.8 mM EDTA	3.0
		0.8 mM EDTA + 0.2 μg/ml valinomycin	4.2

The proton conductivity in wild-type mitochondria was slightly higher than in mitochondria isolated from the respiration-deficient mutant. The proton conductance coefficients were 5 to 10 times higher than in rat-liver mitochondria[13]. The rate of pH equilibration in mitochondria from wild-type and mutant yeast was considerably increased by CCCP in the presence of valinomycin and K^+ (Fig. 3).

Addition of oxygen to an anaerobic suspension of wild-type yeast mitochondria in the presence of K^+ was followed by transient acidification of the medium, similar to observations with rat-liver mitochondria[14]. H^+/O ratios found under various conditions are shown in Table II. In the absence of valinomycin, the values were rather low and in contrast to observations with rat-liver mitochondria, they were not affected by 0.8 mM EDTA or 4 mM Ca^{2+}. This suggests that Ca^{2+} did not provide a "backlash" for outwardly moving protons during the respiratory pulse. This is consistent with the finding that yeast mitochondria cannot transport Ca^{2+} ions[20,32]. In the presence of valinomycin, H^+/O ratios approached 4 with succinate, citrate, or pyruvate *plus* malate. Since the first coupling site is usually not operative in *Saccharomyces* mitochondria[16,21-23], these values are predicted by the chemiosmotic hypothesis[4].

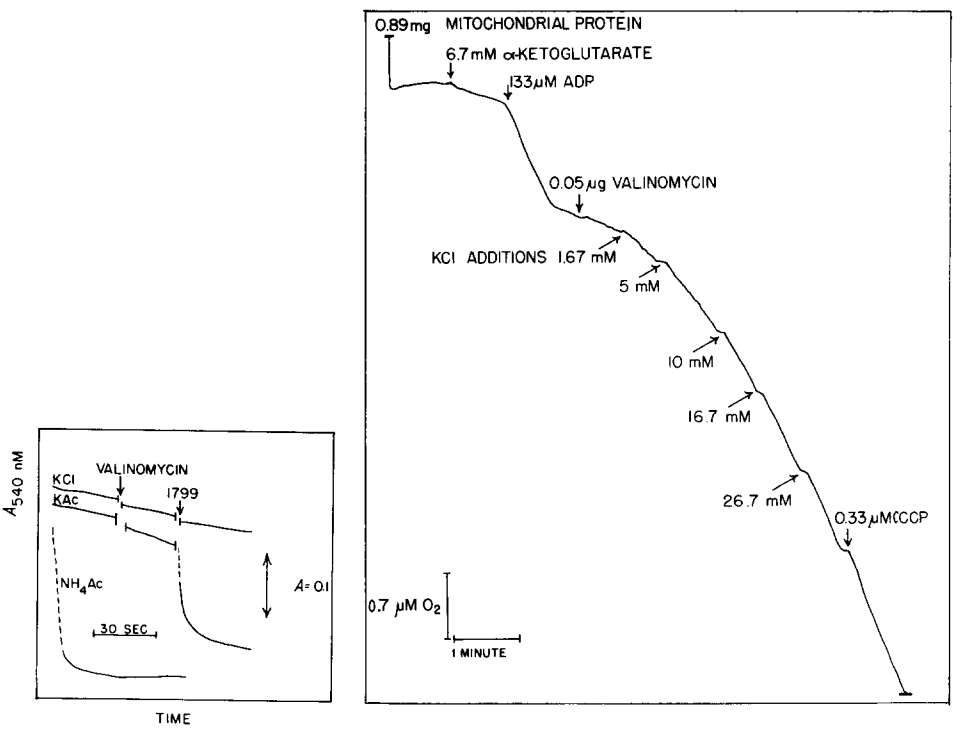


Fig. 4. Passive swelling of yeast mitochondria in isotonic salt solutions. Wild-type yeast mitochondria were suspended at 0.25 mg protein/ml in 0.33 M salt solutions adjusted to pH 6.2 and cont taining 10 mM Tris–maleate (pH 6.2) and 0.2 mM NaCN. Swelling was monitored at 540 nm. A the arrows, valinomycin (0.8 μg/ml) or 1799 (10 μM) were added. KAc, potassium acetate; NH_4Ac, ammonium acetate, 1799, a 2:1 adduct of hexafluoroacetone and acetone, kindly supplied by P. Heytler.

Fig. 5. Effect of K^+ and valinomycin on the respiration of wild-type yeast mitochondria. The O_2 uptake was measured polarographically in 1.5 ml medium containing 0.52 M mannitol and 13.3 mM sodium phosphate (pH 6.5). Further additions were made as indicated in the figure.

Addition of oxygen to anaerobic suspensions of mitochondria from the respiration-deficient mutant did not induce any pH change.

Energy-dependent K^+ translocation in yeast mitochondria

Passive ion uptake in yeast mitochondria was studied with the swelling technique of CHAPPELL AND HAARHOFF[24]. As can be seen from Fig. 4, the mitochondria swelled very rapidly when suspended in ammonium acetate. Rapid swelling in potassium acetate was observed only on addition of both uncoupler and valinomycin. The slow swelling observed in the absence of uncoupler probably reflects a residual H^+ permeability of the mitochondrial membrane. Essentially the same results were obtained when the experiments were carried out with mutant mitochondria. When yeast mitochondria were suspended in 0.6 M mannitol containing 1 to 10 mM K^+ and valinomycin, no swelling with either respiration or ATP as energy source could be observed by the optical procedure. However, valinomycin increased state 4 respiration of yeast mitochondria suggesting a respiration-dependent K^+ transport. As shown in Fig. 5, wild-type yeast mitochondria oxidizing substrate in the absence of K^+ showed typical state 4–state 3 transistion upon addition of a small amount of ADP. Valinomycin was almost without effect on respiration, but subsequent addition of K^+ stimulated respiration. The K_m for K^+, calculated from several experiments, was 8 mM. The maximal rate of respiration in the presence of K^+ and valinomycin (50 μg/g mitochondrial protein) was similar to the rate induced by CCCP.

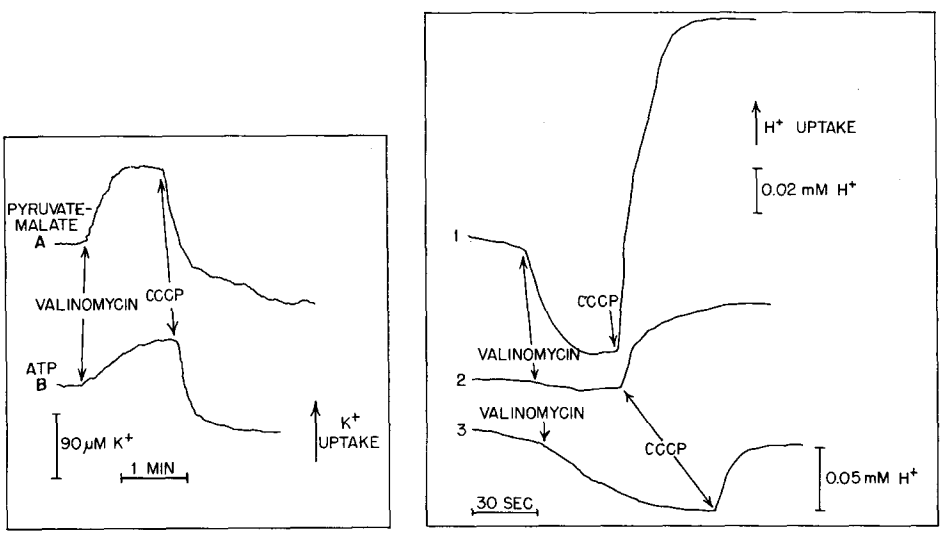


Fig. 6. Potassium transport in yeast mitochondria. Mitochondria from wild-type yeast (3.5 mg protein/ml) were incubated in 0.4 M mannitol, 50 mM choline chloride, 3 mM sodium phosphate and 2 mM KCl. The final pH was adjusted to 6.2. As an energy source was added A: 6.2 mM sodium pyruvate + 6.2 mM sodium malate; or B, 10 mM ATP (sodium salt). At the arrows valinomycin (0.2 μg/ml) or CCCP (2.5 nmoles/ml) were added.

Fig. 7. Effect of valinomycin and CCCP on the energy-linked H^+ translocation in yeast mitochondria. Mitochondria from wild-type yeast (1.5 mg protein/ml) were incubated in 0.59 M mannitol, 2.4 mM sodium phosphate and in (1) 8 mM KCl, 4 mM pyruvate + malate (sodium salts) (pH 5.5); (2) as in (1) but without KCl; (3) 8 mM KCl, 8 mM ATP (pH 6.0). At the arrows, valinomycin (0.24 μg/ml) or CCCP (8 nmoles/ml) were added.

In view of the results with passive ion movements, rather small amounts of K^+ were expected to be taken up. This was found to be the case when K^+ uptake was measured with a K^+-sensitive electrode. Fig. 6 shows uptake of K^+ in wild-type yeast mitochondria supported by respiration or ATP hydrolysis in the presence of valinomycin. The amount of K^+ (18 μg/g) taken up was only 5 % of that which can be taken up by rat liver mitochondria (390 μg/g) under similar conditions[25,26]. This difference might be at least partly accounted for by the smaller volume of isolated yeast mitochondria compared with rat-liver mitochondria. The Michaelis constant for K^+ uptake was calculated to be approx. 10 mM and the maximal rate of uptake about 125 μg K^+ per min per g protein. As can be seen from Fig. 6, both the rate and the extent of K^+ transport were higher when respiration of pyruvate plus malate rather than ATP was used as source of energy.

K^+ movement across the mitochondrial membrane is usually accompanied by countermovement of H^+ (refs. 25–28). In yeast mitochondria, where the low extent and high Michaelis constant limit the measurement of K^+ translocation, H^+ efflux induced by valinomycin in a K^+-rich medium in the presence of substrate or ATP can be conveniently used as an indirect measurement of K^+ transport (Fig. 7). Diminishing tonicity of the suspending medium or replacement of mannitol by choline chloride inhibited both the rate and the extent of the valinomycin-induced H^+ efflux. Substitution of phosphate by acetate did not affect the extent of H^+ efflux but the rate was diminished.

Fig. 8 shows the effect of valinomycin on H^+ and K^+ translocation in aerobic suspension of mitochondria from the respiration-deficient mutant supplemented with ATP. The slight continuous acidification of the medium at higher pH probably represented ATP hydrolysis[29] and was not affected by valinomycin or CCCP. As may be seen from the trace of the K^+-sensitive electrode, valinomycin addition did not induce K^+ uptake but rather a slow and small leakage of K^+ from the mutant mitochondria.

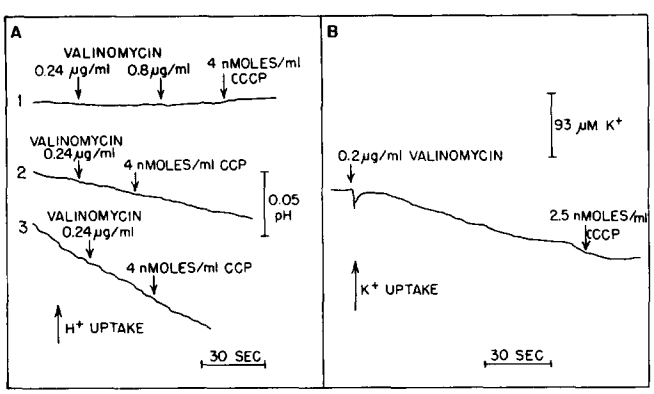


Fig. 8. The absence of valinomycin-induced K^+ uptake and H^+ release in mutant yeast mitochondria. A: Mutant yeast mitochondria (1.45 mg protein/ml) were incubated in 0.59 M mannitol, 2.4 mM Na phosphate, 80 mM KCl and 8 mM ATP. The pH was adjusted to; (1) 6.0; (2) 6.9; (3) 7.3. Valinomycin and CCCP were added at the arrows as indicated. B: Mutant yeast mitochondria (4.05 mg protein/ml) were incubated in 0.48 M mannitol, 50 mM choline chloride, 3 mM sodium phosphate, 2 mM KCl, 8 mM ATP at pH 6.9. Valinomycin and CCCP were added as indicated.

These results suggest that ATP hydrolysis by the mutant mitochondria could not support efflux of H^+ or uptake of K^+. It was therefore of interest whether other energy-dependent reactions are present in mutant mitochondria.

ANS fluorescence and $^{32}P_i$–ATP exchange in yeast mitochondria

The fluorescence of ANS can be affected by the energy state of mitochondria[10] or submitochondrial particles[9]. Addition of an oxidizable substrate or of ATP results in a decrease of fluorescence in mammalian mitochondria[10] and an enhancement of fluorescence in submitochondrial particles[9].

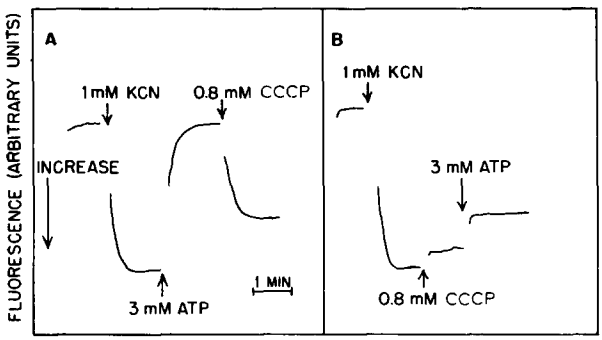
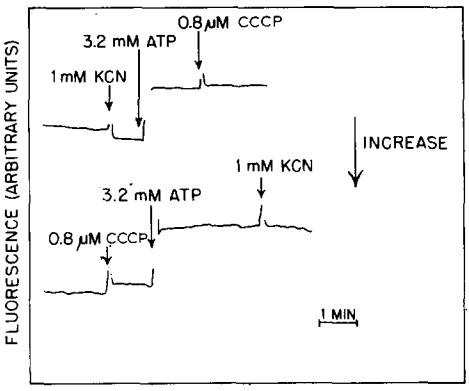


Fig. 9. Changes in ANS fluorescence in wild-type yeast mitochondria. The incubation mixture contained 0.6 M mannitol, 20 mM Tris–HCl (pH 7.5) and 30 μM ANS. Fluorescence was measured as described. Additions were made as indicated.

In order to exclude any response of ANS to electron transport, the fluorescence measurements were carried out in the presence of KCN (Fig. 9). Addition of KCN to wild-type yeast mitochondria resulted in an increase in fluorescence (Fig. 9A). Upon addition of ATP, the fluorescence diminished abruptly and then continued to decrease for several minutes. Subsequent additions of CCCP (in a concentration as low as 0.8 μM) induced a rapid increase in fluorescence. If CCCP was added prior to ATP, the fluorescence was only slightly decreased; ATP added subsequently led to the immediate drop in fluorescence but the slow decreasing phase was not observed (Fig. 9B). In mutant mitochondria, KCN and CCCP did not significantly affect ANS fluorescence; with ATP, the initial rapid drop was observed but the subsequent uncoupler-sensitive decrease in fluorescence, indicative of the formation of the high-energy state did not take place (Fig. 10).

Very low $^{32}P_i$–ATP exchange activity was found in the mutant mitochondria under conditions optimal for the exchange reaction in wild-type yeast mitochondria (Fig. 11). The specific activities of this exchange varied between 1.6 and 3.1 μmoles P_i per min per g protein compared with 143 μmoles P_i per min per g protein in wild-type yeast mitochondria. Unlike the exchange reaction catalyzed by wild-type yeast mitochondria, the exchange activity of the mutant mitochondria was insensitive to uncoupling agents, oligomycin and atractyloside (Figs. 11 and 12). This indicated that the low residual activity of the mutant mitochondria did not represent a partial reaction of oxidative phosphorylation. It is known that glyceraldehyde-3-*P* dehydroge-



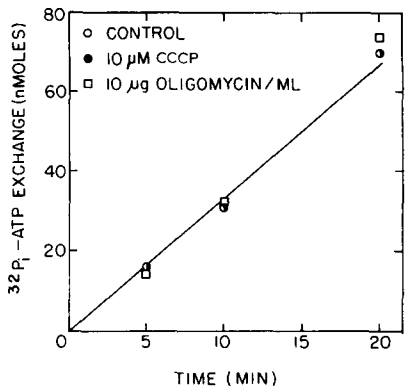


Fig. 10. Changes in ANS fluorescence in mutant yeast mitochondria. Incubation mixtures were as described in Fig. 9.

Fig. 11. Time course of the P_i–ATP exchange in mutant yeast mitochondria. The P_i–ATP exchange reaction was measured as described before[11] using 1 mg mitochondrial protein per ml. Additions were made as indicated.

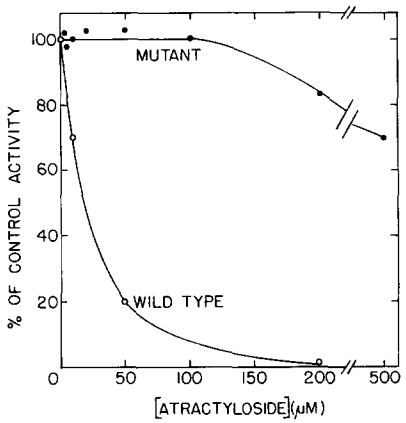


Fig. 12. Effect of atractyloside on the P_i–ATP exchange reaction in mutant and wild-type yeast mitochondria. The P_i–ATP exchange reaction was measured as described.

nase and 3-phosphoglycerate kinase in the presence of their substrates catalyze a $^{32}P_i$–ATP exchange[30]. The exchange reaction exhibited by mutant mitochondria was in fact stimulated 2-fold by a mixture of glyceraldehyde-3-*P* and 3-phosphoglycerate and was inhibited by 1 mM iodoacetate. Glyceraldehyde-3-*P* dehydrogenase activity (145 μmoles NAD^+ reduced per min per g protein) was detected in a suspension of the mutant mitochondria.

All these findings do not support the recent claim[31] that energy-dependent K^+ uptake and even oxidative phosphorylation are preserved in mitochondria isolated from a cytoplasmic respiration-deficient mutant.

It may be concluded that the cytoplasmic "petite" mutation inactivates both the respiratory chain and the energy-transfer mechanism. Although the mitochondrial membrane of the respiration-deficient mutant would still be capable of maintaining

the separation of electric charges, the energy of ATP hydrolysis can no longer be channeled into the charge separation system.

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